IN VITRO METHODS AND THE CHALLENGE OF EXCEPTIONAL SPECIES FOR TARGET 8 OF THE GLOBAL STRATEGY FOR PLANT CONSERVATION[1]

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ABSTRACT

For the majority of plant species of conservation concern, seed banking and traditional propagation methods are the most efficient ways of meeting the ex situ and recovery conservation goals of Global Strategy for Plant Conservation (GSPC) Target 8. However, there are estimated to be 5000 or more endangered species for which these methods will not be adequate conservation tools. These "exceptional" species are those with recalcitrant seeds or those that produce few or no seeds. In vitro methods can provide alternative procedures for propagating and preserving germplasm in the long term for these species. Research at the Center for Conservation and Research of Endangered Wildlife (CREW) with several U.S. endangered species has shown the potential of these methods. In vitro propagation can provide plants for reintroduction and research when traditional propagation methods are not adequate. Phytotissue banking can be used for long-term ex situ conservation when seed or embryo banking is not possible. In vitro methods are also needed for recovery when embryo banking of recalcitrant seeds is possible. The full implementation of in vitro methods is constrained by information, scientific, and economic challenges, but the need for its use in meeting the needs of exceptional species should provide impetus for overcoming these challenges and making these methods an integral part of an overall ex situ conservation strategy.

Key words: Cryopreservation, ex situ conservation, Global Strategy for Plant Conservation (GSPC), in vitro, tissue culture.

Ex situ conservation has become a major tool for plant conservation. As such, it is one of the major targets of the Global Strategy for Plant Conservation (GSPC). Target 8 states: "At least 75 per cent of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20 per cent available for recovery and restoration programmes" (Convention on Biological Diversity [CBD], 2011). This goal is primarily accomplished through seed banking, which, for most species, provides the most efficient method for long-term preservation of multiple genotypes. However, there is a subset of endangered species for which seed banking is not workable, including species with recalcitrant seeds (desiccation-sensitive seeds that cannot survive traditional seed banking protocols) and a significant number of species that produce few or no seeds, or for which seed collection is not practical. Combined, these form a group of unbankable or "exceptional" species that may comprise an estimated 5000 to 10,000 plant species of conservation concern (Pence, 2011a). Because exceptional species are fewer in number than species with available, bankable seeds, and because they require more costly approaches to ex situ conservation, they are more likely to be overlooked as a group. However, these exceptional species are just as likely, if not more so, to hold potential value, since many are components of species-rich and ecologically complex tropical rainforests. Thus, it is important to consider the challenges posed by this group for conservation biologists and to evaluate what is needed for dealing with them.

IN VITRO TOOLS IN EX SITU CONSERVATION AND RESTORATION

Many of the tools available for dealing with the ex situ conservation and propagation of exceptional species include at least some aspects of in vitro culture, and the extent to which this biotechnology is needed will influence the labor and resources required for a particular species (Pence, 2011a, 2011b). For example, excised embryo axes from recalcitrant seeds, such as the endangered Texas wild-rice *Zizania texana* Hitchc. and the common horse chestnut, *Aesculus hippocastanum* L., have been shown to survive rapid freezing in liquid nitrogen (Wesley-Smith et al., 2001; Walters et al., 2002). This allows the cryobanking of multiple genotypes in one sample, as is the case with intact

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seeds. However, this requires the excision of embryos prior to banking followed by the recovery of the embryos using in vitro systems, thereby increasing the input of labor and resources compared with seed banking. Another approach is the cryopreservation of dormant buds in woody taxa, which has been demonstrated with cold-hardy and cold-acclimated tissues (Towill & Ellis, 2008). With some plant species, such as willows (*Salix* L.) and apples (*Malus* Mill.), recovery can be accomplished through bud grafting or direct rooting (Forsline et al., 1998; Towill & Widrlechner, 2004), while in vitro methods have been used in persimmon *Diospyros kaki* Thunb. for post-freezing recovery growth (Matsumoto et al., 2001). If embryo axes or buds of an exceptional species cannot be cryopreserved, tissue banking using in vitro methods may be the more effective route for securing germplasm ex situ.

In vitro tools for conservation and restoration are based on the growth of plant tissues in tissue culture, a flexible technique that can adapt to a variety of needs (George & Debergh, 2008). The primary requirement for ex situ conservation is that plants can be recovered for restoration needs. Shoot propagating cultures and somatic embryo cultures are both capable of producing plants in vitro that can be acclimatized to soil and used for restoration. Shoot tips and embryogenic cultures have also been used for cryopreserving tissues long term in liquid nitrogen (Lombardi et al., 2008; Reed, 2008a), and thus, these have been the primary types of cultures used in conservation efforts.

In vitro propagation protocols have been described for hundreds of plant species, many of which are of economic importance, but some protocols have been developed for endangered taxa, such as several from Australia, Spain, and Hawaii (Bunn, 2003; Gonzalez-Benito & Martin, 2011; Sugii, 2011). In vitro methods can assist plant conservation starting at the point of a taxon's collection. The technique of in vitro collecting can be used to initiate tissue cultures in the field, when seeds or cuttings are unavailable or unworkable (Pence & Engelmann, 2011). In vitro propagation can be used to maintain ex situ collections, as is being done for native Brazilian vascular taxa (Pilatti et al., 2011) and for European bryophytes (Rowntree et al., 2011). Tissue culture can also be used to produce plants for restoration, as has been done with the Autumn buttercup *Ranunculus aestivalis* (L. D. Benson) Van Buren & K. T. Harper and the Cumberland sandwort *Minuartia cumberlandensis* (Wofford & Kral) McNeill. Both are two endangered species in the United States that have been propagated through tissue culture and outplanted in Utah and Kentucky, respectively (Pence et al., 2008a, 2008b). By using in vitro propagated plants, restoration experiments can be conducted without loss of genetic material, since back-up material of each clonally propagated line can be maintained in the lab, either as an active culture or as cryopreserved tissue. This may be particularly useful in seeking out new habitat if assisted migration experiments are undertaken with exceptional species (Vitt et al., 2010).

In vitro cultures can also provide material for cryopreserving tissues for long-term ex situ storage (Engelmann, 2011). While fewer species have been cryopreserved, compared with those propagated in vitro, liquid nitrogen storage has been a valuable tool for maintaining germplasm of rare plant taxa, particularly those classified as exceptional species. Tissue cryopreservation has been vital for the ex situ conservation of the federally listed Todson's pennyroyal, *Hedeoma todsenii* R. S. Irving, from New Mexico, for which seed production has never been observed (Pence et al., 2009). Species management utilizing long-term storage in liquid nitrogen has been effectively used across major plant groups ranging from European bryophytes (Rowntree et al., 2011) to vascular plants under threat in the native Australian flora (Kacmarczyk et al., 2011). Several basic methods are available and cryopreservation appears to be adaptable to a wide range of plant species (Reed, 2008a), although methods often need to be adjusted for particular species and genotypic variations. As with in vitro propagation, work with a related but common species can often facilitate protocol development for rarer species (Turner et al., 2001).

Challenges for the Application of In Vitro Methods

It is clear that in vitro tools allow us to go beyond the limits of traditional seed banking to include exceptional species in conservation efforts, making it possible to include, in theory, almost all plant species within the focus of Target 8 of the GSPC. Work in translating this theory into practice, however, faces at least three challenges.

THE INFORMATION CHALLENGE

Fundamental to addressing the needs of exceptional species is the challenge of identifying those species and determining the most effective and efficient methods for their conservation. While it is possible to develop a rough estimate of the number of exceptional species that may exist (Pence, 2011a), identifying them for specific conservation efforts will require at least three levels of information. The first requirement is the comprehensive listing of all plant species set as Target 1 of the GSPC. This has been largely completed with ongoing updates by the Missouri Botanical Garden and the Royal Botanic Gardens, Kew, and can be accessed online (The Plant List, 2010). Second, within this list, species that are of conservation concern must be identified, as defined in Target 2 of the GSPC. Work on this has been conducted on many fronts, as Red Lists have been compiled, often at regional or national levels. The global effort is spearheaded by the International Union for Conservation of Nature and Natural Resources (IUCN, 2011) and is in progress.

Finally, the third need is to define for each endangered species the most appropriate methods for achieving the ex situ conservation and restoration goals of Target 8 of the GSPC. This is the most challenging level of information to acquire, since this requires an evaluation of whether the species can be stored conventionally or is an exceptional species. Some of this information is becoming available as species are described in national or regional lists of endangered plants, such as in the Plant Profiles for the Center for Plant Conservation's (CPC) National Collection of Endangered Plants (CPC, 2012). Information on seed characteristics for both endangered and non-endangered species is also being compiled in the Seed Information Database (Royal Botanic Gardens Kew, 2008). However, because this will depend on having a completed list of endangered plant species and will require the individual evaluation of each of those species, a full listing of endangered exceptional species is not yet available.

Despite this lack of information, many species are already known that fall into the exceptional category, and some of these are known to be endangered (CPC, 2012). Methods for tissue banking have been applied to several exceptional species in this laboratory to examine the logistics and costs of dealing with cryopreserving multiple genotypes for ex situ conservation (Pence et al., 2009; unpubl.). These species include three federally endangered exceptional species: the fourpetal pawpaw, *Asimina tetramera* Small, and Avon Park rattlebox, *Crotalaria avonensis* DeLaney & Wunderlin, which are both found in the state of Florida, as well as Todsen's false pennyroyal, *Hedeoma todsenii*, from New Mexico. Species that are already known as exceptional can serve as models for evaluating and refining methods that can then be applied to the larger group of species, yet to be identified.

SCIENTIFIC CHALLENGES

Plant tissue culture, while firmly based in the science of plant physiology and development, still encompasses a wide range of variability between species and often among genotypes. Developing protocols for species new to tissue culture is normal for those wishing to use in vitro methods for plant conservation. Some species respond easily to commonly used protocols (George & Debergh, 2008), while other taxa may prove recalcitrant to one or more of the steps necessary to culture the plant and return it to ex vitro conditions. Species may be difficult to initiate into culture due to internal contamination, excessive production of phenolic compounds, or unusually slow growth. Shoots may be difficult to root; somatic embryos may not develop into normal plants in vitro; and plants may not acclimatize easily from culture to soil substrates (Benson, 2000; Bunn, 2003; Bunn et al., 2011). All of these may be points of challenge, depending on the species. Even closely related species and genotypes within a single exceptional species can be more or less amenable to the various steps of culture (Reed, 2008b; Uchenda & Reed, 2008). The goal of any in vitro work with an endangered taxon is to efficiently and effectively move it into culture, to preserve plant tissue, and ultimately to propagate plants from in vitro cultures for restoration projects. While this is rarely achieved with ease, there are species that can serve as models for directions that researchers might explore in dealing with more difficult species.

Species of *Saintpaulia* H. Wendl., or African violets, are endangered in their native habitat of southern Kenya and northern Tanzania (Eastwood et al., 1998), despite the fact that *Saintpaulia* hybrids are common worldwide as houseplants. *Saintpaulia* is easily propagated, even without tissue culture intervention, but in vitro propagation can provide plants free from pests and diseases that might be more suitable for restoration projects than conventionally propagated plants. In vitro cultures can also provide material for tissue cryopreservation and banking when seeds are not available at the time that a wild habitat site is visited. *Saintpaulia* leaves will survive several days of common postal transport or courier, and thus, leaves can be collected and sent to a distant lab for culture. *Saintpaulia* shoots can be regenerated from leaf tissue using several media formulations. These shoots root easily in culture and the resulting plants acclimatize readily (e.g., Cooke, 1977; Mithila et al., 2003; Khan et al., 2007). Shoot tips from in vitro cultures of commercial cultivars of *S. ionantha* H. Wendl., originally native to Tanzania and Kenya, have been shown to survive three of the standard cryopreservation protocols (Moges et al., 2004). Normally, one of the most labor-intensive steps in shoot tip cryopreservation is the dissection of the many small shoot tips needed. However, with *Saintpaulia* on some hormonal combinations, many small buds form as clusters on the cut margins of cultured *Saintpaulia* leaves. We have found that shoot clusters can be scraped from the leaf surface with a scalpel, cut into smaller pieces, and then subjected to cryopreservation procedures. While each meristem is not completely delineated in this system, the growing points are small enough and numerous enough to provide material that can survive liquid nitrogen exposure and later regrow (Fig. 1). Such a system greatly reduces dissection time and, consequently, the labor involved in cryopreservation protocols. The ability to manipulate growth in vitro in other species to provide similar tissue for cryopreservation could help facilitate the ex situ conservation of exceptional species.

An approach that has been commonly used when dealing with new species in culture is to use information from the culture of related, but non-endangered species to guide the development of new protocols. This approach was used in recent work with four endangered oak (*Quercus* L.) species from the United States. Methods used in other laboratories to initiate cultures and produce shoots and somatic embryos with several nonthreatened species ultimately proved successful with three of four species tested that were new to culture, *Q. arkansana* Sarg., *Q. boyntonii* Beadle, and *Q. georgiana* M. A. Curtis (Kramer & Pence, 2012). However, this approach may not always be possible, as many endangered species do not have congeners that have been grown in vitro, and families with high numbers of endangered species have not always been prioritized for in vitro studies (Pence, 2011b). Other avenues could also prove helpful. Natural adaptations have been recognized as an important consideration in developing cryopreservation protocols (Benson, 2008), and these may also prove to be important in developing in vitro propagation protocols for species new to culture.

THE CHALLENGE OF COSTS

The third major challenge for efforts directed at meeting Target 8 for exceptional species is the cumulative cost, a factor closely linked to the information and scientific challenges described above. This subject has been discussed in more detail elsewhere (Pence, 2011a), but in general, labor is the primary cost for protocols involving in vitro methods. While the costs are always likely to be higher than seed banking and traditional propagation methods, the more readily a species is initiated into culture, cryopreserved, and propagated, the less expensive those efforts will be. Information on the requirements of individual species is also critical, as different exceptional species will require varying levels of attention. For example, species that can be banked as isolated embryos will avoid the costs of initiating an in vitro culture before cryopreservation and will include multiple genotypes in a single banking exercise. Species requiring tissue banking must be initiated into culture, cryopreserved as multiple genetic lines, and recovered in vitro, thereby increasing efforts and costs. However, once banked, maintenance costs for tissues or embryos in liquid nitrogen are lower than maintaining species as active in vitro cultures or in living collections and do compare favorably with seed banking (Epperson, 1997; Reed et al., 2004; Keller et al., 2008; Li & Pritchard, 2009).

Meeting the increased costs of propagating and preserving exceptional species will benefit from multiple levels of collaboration and coordination. Academia, nonprofit organizations, government, and industry all have expertise and infrastructure that might be utilized in meeting Target 8 for exceptional species by contributing to basic research, protocol development, and actual banking or propagation activities. Over the long term, the cost of banking and maintaining endangered exceptional species ex situ will be significantly lower than the costs of comparably maintaining ex situ living collections of endangered animals, but the costs for exceptional species as a group will likely be measured in millions of dollars (Pence, 2011a). The challenge of these costs will best be minimized by creatively using multiple partners, coordinating information and effort, harnessing basic research, and leveraging monetary funding with in-kind support.

CONCLUSIONS

Exceptional species, or those without bankable seeds, pose a significant challenge to meeting Target 8 of the GSPC. Representing perhaps 5% or less of all endangered species, the goal in Target 8 of "at least 75 per cent of all threatened plant species in ex situ collections," and "at least 20 per cent available for recovery and restoration programmes" (CBD, 2010; Sharrock et al., 2010) could be met by not including any exceptional species. However, the worth of a species to humankind and to the environment cannot be determined by its ability to conform to traditional ex situ conservation methods.

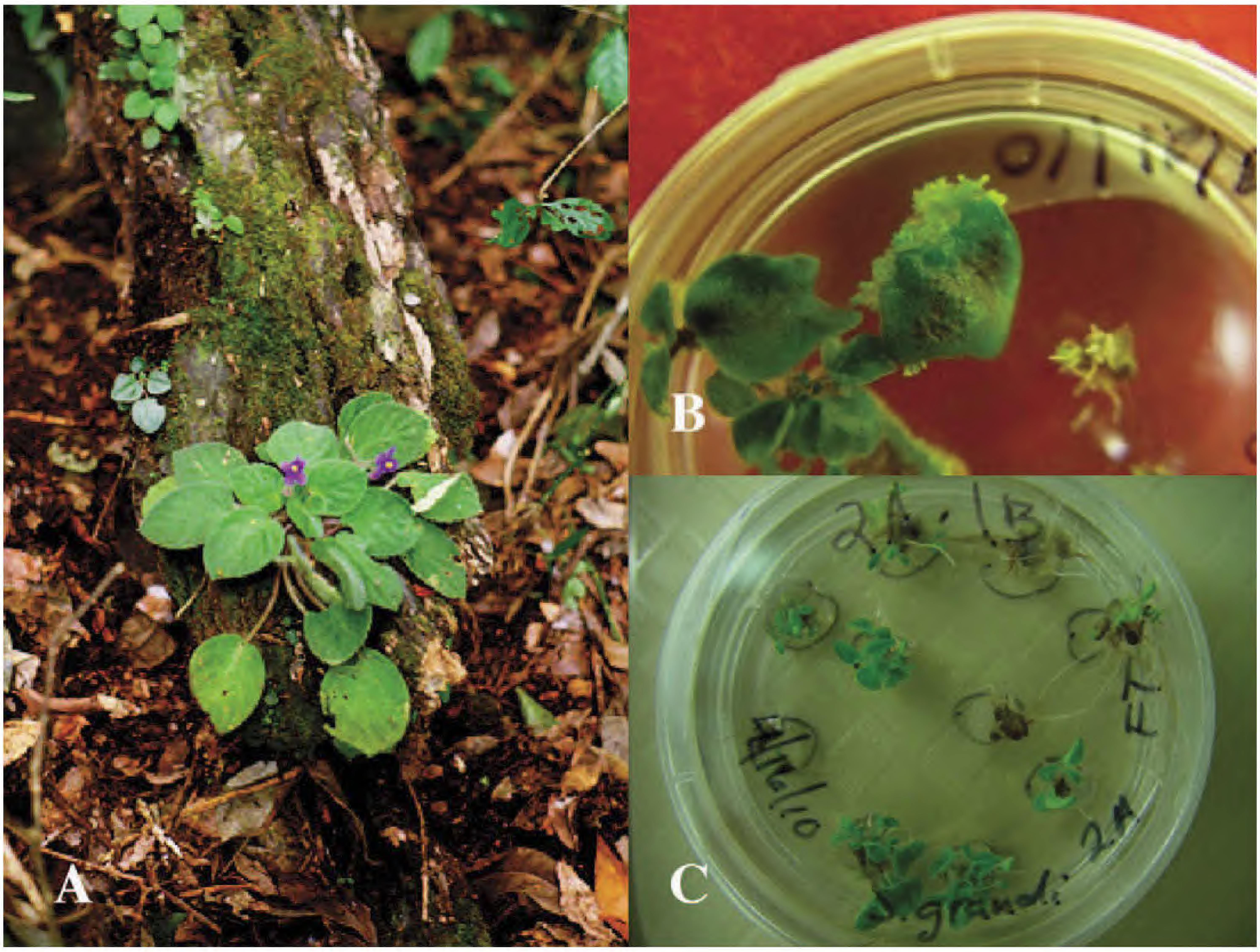

Figure 1. —A. *Saintpaulia* plant growing in Tanzania. Photo by Johanna Kolehmainen. —B. Adventitious buds of *Saintpaulia ionantha* H. Wendl. subsp. *rupicola* (B. L. Burtt) I. Darbysh. forming on leaf cultured in vitro. —C. Cryopreserved buds of *S. ionantha* subsp. *grandifolia* (B. L. Burtt) I. Darbysh. growing into small plants on recovery medium. This culture was from African violet stock that originally came from Tanzania. Images taken by V. C. Pence at the Center for Conservation and Research of Endangered Wildlife (CREW).

Thus, consideration needs to be given to what resources and approaches are needed to include highly threatened exceptional as well as conventional species in efforts to achieve Target 8.

This challenge needs to be met by the use of alternative tools, including in vitro methods, but will require information that identifies the needs of particular species for these tools. The effective use of in vitro methods will also require further research into the basic science of manipulating plants in tissue culture as well as the development of coordinated collaboration between multiple partners with complementary skill sets, in order to most effectively and efficiently utilize existing expertise and infrastructure. These challenges are significant, but the need for conserving all endangered plant species mandates that they be addressed. By identifying and understanding these challenges, they will be more easily evaluated and integrated into strategies for the ex situ conservation and restoration of all rare plant species into the future.

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